DISTANT N-TERMINAL AND C-TERMINAL DOMAINS ARE REQUIRED FOR INTRINSIC KINASE ACTIVITY OF SMG-1, A CRITICAL COMPONENT OF NONSENSE-MEDIATED mRNA DECAY

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Running Title: Structure and Activity of SMG-1

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Phosphatidylinositol 3-kinase-related kinases (PIKKs) consisting of SMG-1, ATM, ATR, DNA-PKcs, and mTOR are a family of proteins involved in the surveillance of gene expression in eukaryotic cells. They are involved in mechanisms responsible for genome stability, mRNA quality, and translation. They share a large N-terminal domain and a C-terminal FATC domain in addition to the unique serine/threonine protein kinase (PIKK) domain that is different from classical protein kinases. However, structure-function relationships of PIKKs remain unclear. Here we have focused on one of the PIKK members, SMG-1 that is involved in RNA surveillance termed nonsense-mediated mRNA decay (NMD), to analyze the roles of conserved and SMG-1 specific sequences on the intrinsic kinase activity. Analyses of sets of point and deletion mutants of SMG-1 in a purified system and intact cells revealed that the long N-terminal region and the conserved leucine in the FATC domain were essential for SMG-1 kinase activity. However, the conserved tryptophan in the TS domain and the FATC domain was not. In addition, the long insertion region between PIKK and FATC domains was not essential for SMG-1 kinase activity. These results indicated an unexpected feature of SMG-1, i.e., the distantly located N-terminal and C-terminal sequences were essential for the intrinsic kinase activity.

SMG-1 was first identified as a suppressor of morphogenetic effect on genitalia-1 in *Caenorhabditis elegans*. It is one of the critical components of the RNA surveillance pathway termed nonsense-mediated mRNA decay (NMD), that is conserved from worm to human (1-4). NMD mediates rapid degradation of mRNAs bearing premature termination codons (PTCs) generated by genome mutations and by errors that occur during processes including transcription and splicing (5). NMD removes aberrant mRNAs containing PTCs from cells, thereby protecting them from accumulation of non-functional or potentially harmful truncated proteins encoded by aberrant mRNAs. In mammals, SMG-1 directly phosphorylates Upf1, a central component of NMD, at the serine/threonine-glutamine-rich (S/T-Q rich) motif, in the carboxyl terminal of Upf1, followed by dephosphorylation by PP2A (3,6,7). This phosphorylation/dephosphorylation cycle of Upf1 is essential to promote NMD. In addition to RNA surveillance, a recent study suggested the possible involvement of SMG-1 in genome surveillance in mammalian cells (8).

SMG-1 is the newest member of the family of phosphatidylinositol 3-kinase-related protein kinases (PIKKs) that includes mTOR (mammalian target of rapamycin), ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-

related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) in mammalian cells. PIKKs have intrinsic serine/threonine kinase activity, and are distinguished from other kinases by their unique catalytic domain (PIKK domain) similar to lipid phosphatidylinositol 3-kinase (PI3K) catalytic domain and their huge molecular weight (270-470 kDa) (9-11) SMG-1, ATM, ATR, and DNA-PKcs are also termed S/T-Q-directed kinases, based on their strong preference for phosphorylating serine (S)/threonine (T) followed by a glutamine (Q) (3,12). PIKKs function in diverse cellular processes including genome surveillance (ATM, ATR, and DNA-PKcs) (9,11), mRNA surveillance (SMG-1) (1,4), and translation control (mTOR) (10). PIKKs are also characterized by their highly conserved C-terminal region termed the FATC (FRAP, ATM, TRRAP C-terminal) domain (13). Except for SMG-1, the FATC domain is located nearby the PIKK domain, and is critical for basal kinase activities of mTOR and DNA-PKcs (14-16) or the activation of ATM by extracellular stimulation (17). Although sequences are quite different among family members, the N-terminal region of PIKKs including that of SMG-1 seems to have a similar structure. Computational and three-dimensional analyses predicted that N-terminal regions of DNA-PKcs, ATM, ATR, and mTOR contained many helical repeats such as HEAT (huntingtin, elongation factor, A subunit of PP2A, TOR1), TPR (tetraticopeptide repeat), and ARM (armadillo repeat motif) repeats (18,19).

In addition to common conserved features in PIKKs, SMG-1 has unique sequences. The TS (TOR, SMG-1 homology) domain, which we renamed from the FRBH (FKBP12-rapamycin binding homology) domain, contains the region which shows similarity with the FRB domain of mTOR (supplementary Fig. 1*A*) (3). However, there is no evidence suggesting that SMG-1 interacts with the FKBP-rapamycin complex (2,3). Furthermore, the N-terminal region of SMG-1 shows unique conserved sequences (OCR: one-specific conserved region) among species (OCR1-OCR3; supplementary Fig. 1*B*). In addition, human SMG-1 has a quite large insertion sequence between the PIKK domain and the FATC domain (supplementary Fig. 1*A*) (1,3,20). Although these features suggest involvement of these unique sequences in activity of SMG-1, structure-function relationships of SMG-1 remain completely unknown.

In this study, we investigated structure-activity relationships of human SMG-1. For this purpose, we constructed a variety of point and deletion mutants, and evaluated their kinase activities both *in vitro* and *in vivo*. Our analysis revealed that the FATC domain was important for SMG-1 kinase activity, and the essential amino acid residue for SMG-1 kinase activity was different from that of mTOR, but another conserved amino acid was required. Surprisingly, the N-terminus of SMG-1, which is far from the catalytic domain, strongly affected SMG-1 kinase activity. On the other hand, the long insertion region between the PIKK domain and the FATC domain was not critical for SMG-1 kinase activity. Taken together, this first comprehensive analysis of relationships of kinase activity and structural features of SMG-1 suggested that SMG-1 activity was provided through both the N-terminal region and FATC domains.

Experimental procedures

Plasmid construction – The SR vector based on FLAG-SMG-1 WT has been described previously (7). FLAG-SMG-1 D2331A encoding a kinase-dead mutant of SMG-1 was created by subcloning His-SMG-1 D2331A (3), digested with ApaI/SalI into FLAG-SMG WT digested with ApaI/XhoI. FLAG-SMG-1 L3646A and W3653A: BglII/BamHI fragments encoding the C-terminal region of SMG-1 were subcloned into pBluescript, and point mutations were inserted by site-directed mutagenesis using the Quick Change Kit following the manufacturer's instructions (Stratagene). FLAG-SMG-1 WT was digested with XhoI/NheI to remove the FATC domain, and the XhoI/NheI fragment of subcloned plasmids containing the mutation was inserted in it. FLAG-SMG-1 W1962F: KpnI/EcoRI fragment encoding the TS domain of SMG-1 was subcloned into pBluescript, and point mutations were inserted by site-directed mutagenesis, as described above. FLAG-SMG-1 WT was digested with EcoRV, and the EcoRV fragment of the subcloned plasmid containing the mutation was inserted in it. FLAG-SMG-1 Δ1-152, Δ1-617, Δ1-800, Δ1-998, Δ1-1377, Δ1-1478, Δ1-1708, Δ1-1859, Δ1-2066: a SmaI/XhoI fragment (Δ1-152), SpeI/XhoI fragment (Δ1-617), Eco122I/XhoI (Δ1-800),

HindIII/XhoI fragment (Δ1-998), ApaI/XhoI fragment (Δ1-1377), EcoRV/XhoI fragment (Δ1-1478), KpnI/XhoI fragment (Δ1-1708) or DraI/XhoI (Δ1-152) fragment from FLAG-SMG-1 WT were inserted into SR-FLAG vectors. Δ631-649, Δ631-679, Δ631-791, Δ746-791: SpeI fragment encoding the OCR1 of SMG-1 was subcloned into pBluescript, and PCR-based deletion mutants were constructed using the following primers: for Δ631-649, 5'-tagcgcccacatccctatta-3' and 5'-gctgttcacttccctgccatt-3'; for Δ631-679, 5'-tagcgcccacatccctatta-3' and 5'-tttatctctagtagcctcagtt-3'; for Δ631-791, 5'-tagcgcccacatccctatta-3' and cagagatgtgtcgatgtttgc-3'; for Δ746-791, 5'-tgcgtatgtttcagacttcc-3'and 5'-cagagatgtgtcgatgtttgc-3'. Then, the SpeI fragment of subcloned and deleted plasmids was inserted into FLAG-SMG-1 WT digested with SpeI. Δ994-999: SpeI fragment encoding OCR2 was subcloned into pBluescript and digested by EcoT22I, and self ligated. Then, the BstX1 fragment of deleted subcloned plasmids was inserted into SR-FLAG-SMG-1 WT digested with BstX1. Δ912-967, Δ912-1014: SpeI/HindIII fragment encoding OCR2 was subcloned into pBluescript, and PCR-based deletion mutants were constructed using the following primers; for Δ912-967, 5'-agatgctgtcctttggcag-3' and 5'-ggccatggtaacaaccaac-3'; for Δ912-1014; 5'-agatgctgtcctttggcag-3' and 5'-aatcgccaaacttgtcaggac-3'. Then, the BstX1 fragment of deleted subcloned plasmids was inserted into SR-FLAG-SMG-1 WT digested with BstX1. Δ1375-1477: FLAG-SMG-1 WT was digested by ApaI/HindIII, blunt-ended, and self-ligated. Δ1675-1708: FLAG-SMG-1 WT was digested by Eco52I and EcoRV, blunt-ended, and self-ligated. Δ2771-3254, Δ2771-3359, Δ2513-3490, and Δ2513-3579: BglII/BamHI fragment encoding the C-terminal and 3'UTR region of SMG-1 were subcloned into pBluescript, and PCR-based deletion mutants were constructed using the following primers: for Δ2771-3254, 5'-ggtacaaagggcagaaataata-3' and 5'-cagcgactcaagtgggcagg-3'; for Δ2771-3359, 5'-ggtacaaagggcagaaataata-3' and 5'- actggtcttgaacatcctattg-3'; for Δ2513-3490, 5'- tatttcctccagtagtttgccc-3' and 5'-accttgaaagaactgaaaac-3'; for Δ2513-3579, 5'-tatttcctccagtagtttgccc-3' and 5'-agtgttgcttgtagtcctaaa-3'). Then, the EcoRI/XhoI fragments of the subcloned and deletion plasmid were inserted into FLAG-SMG-1 WT EcoRI/XhoI-digested plasmid.

Cell culture and transfection – HEK293T cells and HeLa TetOff (Clontech) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics, and were incubated at 37 °C with 5% CO_2. For the *in vitro* kinase assay experiments, HEK293T cells (2 x 10^6 cells for the *in vitro* kinase assay, and 1 x 10^7 cells for the kinase amount-dependent *in vitro* kinase assay) were transfected with the indicated FLAG-SMG-1 mutant plasmids using Polyfect (Qiagen) or FuGENE6 (Roche), and cells were collected after 36-48 h.

Depletion of endogenous proteins by RNAi was carried out by using siRNAs as described by Usuki *et al.* (21), except the transfection regents. The following target sequences were used: non-silencing, 5'-aauucuccgaacgugucacgu-3'; SMG-1 3' UTR, 5'-ggaagauuugaugcauucatt'-3'. Non-silencing siRNAs that were guaranteed to show no gene silencing activity were commercially prepared by Qiagen. HeLa TetOff cells were transfected with siRNA duplexes (as above) at a final concentration of 70 nM using RNAifect (Qiagen). At 36 h post-transfection, cells were trypsinized, and re-seeded in 12-well plates. After a further 24 h, cells were transfected with the same siRNAs together with 1 μg of the indicated FLAG-SMG-1 constructs using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after the second transfection.

Protein detection and quantification – Samples were separated by 5.5-12% SDS-PAGE. Western blotting was performed using indicated antibodies with a standard ECL system (GE Biosciences). Anti-SMG-1, Upf1, phospho-Upf1 (3B8) antibodies were generated as previously (3,6), and the anti-FLAG M2 antibody was purchased (Sigma). Coomassie Brilliant Blue (CBB) staining was performed according to standard procedures. To measure amounts of immunopurified mutant proteins from about 150 to 430 kDa, silver staining was used because western blotting efficiency was largely different for each molecular weight of mutants (Supplementary Fig.2). Silver staining was performed using the Silver Quest Silver Staining kit (Invitrogen), following the manufacturer's protocols. Signals were scanned by LAS 3000 (Fuji film), and quantified by Multi Gauge (Fuji

film). Phosphoproteins were detected by autoradiography using BAS 2500 (Fuji film), and signals were quantified as described above.

In vitro kinase assay – FLAG-SMG-1 proteins were immunopurified from transiently transfected HEK 293T cells. Cells were lysed in lysis buffer F (20 mM Tris-HCl at pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM Na orthovanadate, 1 mM Na pyrophosphate, 1 mM NaF, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 100 nM okadaic acid (Calbiochem), and protease inhibitor cocktail (Sigma)), and FLAG-SMG-1 proteins were collected on anti-FLAG affinity gels (Sigma). Immunoprecipitates were washed 4 times in lysis buffer F, and twice in kinase reaction buffer (10 mM Hepes-KOH at pH 7.5, 50 mM ß–glycerophosphate, 50 mM NaCl, 1 mM DTT, and 10 mM $MnCl_2$). Kinase reactions were performed for 15 min at 30°C in 50-μl kinase reaction mixtures (kinase reaction buffer containing 5 μg substrate or GST, 5 μM ATP, and 5 μCi [γ-^{32}P] ATP). As substrate, GST or GST fusion peptides containing Upf1 serine 1078 (3) were used. To measure kinase amount in Fig. 1, immunopurified FLAG-tagged mutants were eluted twice with 50 μl kinase reaction buffer containing 1 mg/ml FLAG peptide for 30 min at 4 °C. The eluted proteins and BSA as control were separated by 7% SDS-PAGE, and visualized by silver staining. Amounts of eluted proteins were calculated from a BSA standard curve, and eluted proteins were used in an *in vitro* kinase assay, as described above.

To detect phosphorylated substrate, the reaction products were separated by 12% polyacrylamide gels and gels were stained with CBB. Gels were dried and phosphoproteins were detected by autoradiography, and signals were quantified as described above. To measure the amount of SMG-1 proteins, the same reaction products were separated by 5.5% polyacrylamide gels, and western blotting (Fig.2 and 4B) or silver staining (Fig.1, 3 and 4A) was performed to detect SMG-1 proteins. After that, the membranes or stained gels were subjected to autoradiography to measure autophosphorylation.

The kinase activity or the autophosphorylation levels of SMG-1 proteins in Fig.2-4 was corrected by phosphorylated signals of substrates or SMG-1 proteins themselves divided by the number of moles of SMG-1 proteins measured as descried above.

Statistical analysis – Statistical analysis were performed by using Student's *t* test.

Results

The FATC domain is essential for SMG-1 kinase activity

To evaluate the intrinsic kinase activity of SMG-1, we immunopurified recombinant SMG-1 from 293T cells transfected with a SMG-1 expression construct, and *in vitro* kinase activity was evaluated using GST-Upf1$^{1072\text{-}1085}$ (3) as a substrate. Previous reports showed that SMG-1 associated with other NMD-related proteins such as Upf1 and SMG-7 (3,7), but immunopurified recombinant SMG-1 did not show any signs of contaminations in silver staining (Fig. 1*B*, lanes 6-10) and western blotting (data not shown), indicating the high purity of the enzyme. As shown in Fig. 1*C* and *D*, substrate phosphorylation depended on amount of FLAG-SMG-1 WT. Furthermore, the fact that FLAG-SMG-1 D2331A, a kinase inactive point mutant in the PIKK catalytic domain (3), did not have any detectable kinase activity supported the validity of the assay. Similar results were also obtained when phosphate incorporation into SMG-1 was evaluated (data not shown). To simplify the assay system, we also performed a kinase assay without elution of FLAG-SMG-1 proteins (Fig.2, see Experimental procedures).

In PIKKs, several point mutations in the region other than the PIKK catalytic domain affect their kinase activity (14-16,22). So we first tested point mutants in which conserved amino acids between SMG-1 and PIKKs were changed using the above kinase assay system. We constructed W1962F point mutant, in which tryptophan at residue 1962 in the TS domain was replaced with phenylalanine. The corresponding residue in mTOR (tryptophan 2027 in mTOR) was critical for kinase activity (Supplementary Fig. 1*A*) (22), however, FLAG-SMG-1 W1962F retained kinase activity and autophosphorylation, and kinase activity was approximately 50% compared to FLAG-SMG-1 WT (Fig. 1, *C* and *D* and Fig. 2, *A* and *B*). This suggested that tryptophan 1962 in the TS domain was not critical for SMG-1 kinase activity, which was different from the results for mTOR.

The next set of point mutants that we used were point mutants (L3646A and W3653F) in the FATC domain, in which conserved leucine or tryptophan between FATC domains in PIKKs were substituted with alanine or phenylalanine essential for the basal/regulatory kinase activity of mTOR (16)/ATM (17) (Supplementary Fig. 1*A*). The L3646A mutant showed greatly reduced kinase activity (7.9% compared to WT). On the other hand, W3653F mutant retained significant activity (50% compared to WT) (Fig. 1, *C* and *D* and Fig. 2, *A* and *B*, black bar). Importantly, similar results were obtained with a different substrate (GST-p53$^{11\text{-}30}$) (data not shown), and with SMG-1 autophosphorylation (Fig. 2, *A* and *B*, white bar.) These results suggested that mutation at the conserved FATC domain strongly affected SMG-1 activity, and that the critical amino acid for kinase activity in SMG-1 was different from those of mTOR and ATM. Since data for kinase activity with or without the elution step were almost similar to each other (Fig.1*D* and Fig. 2*B*), we performed kinase assay without an elution step hereafter.

The long insertion region between the PIKK domain and the FATC domain is not essential for SMG-1 kinase activity

Human SMG-1 contains unique extraordinary large, 1182 amino acids, insertion region between the PIKK domain and the FATC domain (Fig. 1*A*, Supplementary Fig. 1*A*) in contrast to the other PIKKs. Distant FATC domain was essential for its kinase activity as shown above, however, the role of the insertion region remained unknown. Next, we developed deletion mutants FLAG-SMG-1 Δ2771-3254, Δ2771-3359, Δ2513-3490, and Δ2513-3579, lacking different lengths (amino acids 484-1067) of insertion regions. Although about 40-90% of insertion regions were deleted in these mutants, all retained their kinase activity (Fig. 3, black bar, and supplementary Fig.2A), and activities were approximately 32-52% of WT. However, there was no correlation between remaining length of insertion region and kinase activity. We also measured kinase activity for a different substrate, GST-p53$^{11\text{-}30}$, and kinase activities of all mutants for GST-p53$^{11\text{-}30}$ were similar to that for GST- Upf1$^{1072\text{-}1085}$ (data not shown). Interestingly, autophosphorylation level of SMG-1 Δ2513-3490 was about 4 folds higher than that of WT (Fig. 3, white bar, and supplementary Fig.2A). These results indicated that the insertion region unique to human SMG-1 was not essential, but was involved in kinase activity.

N-terminal SMG-1 conserved regions are necessary for kinase activity

The N-terminal half of SMG-1 does not seem to contain any distinct domains, but is predicted to contain many helical repeats (supplementary Fig. 1*B*). To evaluate sequences in the N-terminal region of SMG-1 required for kinase activity, we firstly developed N-terminal deletion mutants FLAG-SMG-1 Δ1-152, Δ1-617, Δ1-800, Δ1-998, Δ1-1375, Δ1-1475, Δ1-1707, Δ1-1858, and Δ1-2067, and measured their kinase activities, as described above (Fig. 4*A*, black bar, and supplementary Fig.2*B*). Intriguingly, all mutants except for Δ1-152 showed a great reduction in kinase activity. Only Δ1-152 retained 50% kinase activity compared to WT. Similar experiments using a different substrate (GST-p53$^{11\text{-}30}$) gave very similar results to those obtained for GST- Upf1$^{1072\text{-}1085}$ (data not shown). Furthermore, mutants except for Δ1-617 showed no significant autophosphorylation (0.02-0.3%) (Fig. 4*A*, white bar, and supplementary Fig.2*B* : see discussion). These results indicated that the end of the N-terminal region of SMG-1 containing OCR1 was extremely important for kinase activity.

To further investigate the region essential for kinase activity within the N-terminal region of SMG-1, we constructed a set of internal deletion mutants: FLAG-SMG-1 Δ631-649, Δ631-679, Δ631-791, Δ746-791, Δ994-999, Δ912-967, Δ912-1014, Δ1375-1477, and Δ1675-1708. These constructs were expressed in 293T cells, but expression of Δ631-649, Δ631-679, Δ994-999 and Δ912-967 tended to be lower than that of WT (data not shown). Unexpectedly, only Δ1675-1708 retained significant kinase activity similar to WT, and other mutants showed greatly reduced activity (Fig. 4*B*, black bar). Δ631-649 with only 19 deleted amino acids in OCR1 and Δ994-999 with only 6 amino acids in OCR2 showed kinase activity of about 8% and 13% of WT, respectively. Similar results were obtained for autophosphorylations (Fig. 4*B*, white bar).

Taken together, these results indicated that the N-terminal half of SMG-1 was generally essential for kinase activity.

***In vivo* kinase activity of SMG-1 mutants**

We examined whether the sequence

required for intrinsic kinase activity of SMG-1, as described above, was required for phosphorylation of Upf1 *in vivo*. For this purpose, we established an *in vivo* assay system to evaluate phosphorylation of endogenous Upf1 by exogenous FLAG-SMG-1 protein in HeLa TetOff cells. We cotransfected siRNAs targeted to the 3'UTR of SMG-1 (21) and FLAG-SMG-1 WT, which do not have SMG-1 3'UTR, into HeLa TetOff cells. As shown in Fig. 5, knockdown of endogenous SMG-1 resulted in suppression of Upf1 phosphorylation to 32% of that in control cells transfected with control siRNA (Fig. 5, *A* and *B*, lanes 1 and 2). Importantly, cotransfection of siRNAs and FLAG-SMG-1 WT could restore Upf1 phosphorylation at 70%, whereas coexpression of inactive kinase mutant FLAG-SMG-1 D2331A couldn't (Fig. 5, *A* and *B*, lanes 3 and 4), indicating that this system could be used to evaluate kinase activity of exogenous SMG-1 against endogenous Upf1 *in vivo*. Thus, we used this assay system to evaluate the activities of some characteristic SMG-1 mutants, i.e., Δ1-617, Δ631-649, Δ746-791, Δ912-1014, Δ1375-1477 and Δ1675-1708. Consistent with *in vitro* kinase activity assays, Δ1675-1708 mutant that retained kinase activity *in vitro* could rescue Upf1 phosphorylation (Fig. 5, *A*, lanes 9 and *B*, lanes 9). On the other hand, Δ1-617, Δ631-649, Δ746-791, Δ912-1014, and Δ1375-1477, which have little or no kinase activity *in vitro*, could not restore sufficient Upf1 phosphorylation *in vivo* (Fig. 5, *A* and *B*, lanes 5-8 and 10). These results showed that SMG-1 mutants that retained kinase activity *in vitro* corresponded to SMG-1 mutants that kept kinase activity *in vivo*.

The N-terminal end of SMG-1 is required for the interaction with SMG-7

To evaluate the role of the N-terminal region of SMG-1, we next examined the interaction between SMG-1 and its known partner proteins, Upf1, Upf2, and SMG-7 (7). Upf1 can independently and directly interact with both the N-terminal half (amino acids 1-2223) and C-terminal half (amino acids 2068-3657) of SMG-1, whereas Upf2 can interact directly with the C-terminal half of SMG-1 (7). Upf2 connects the link between the SMG-1 and EJC, which are required for phosphorylation of Upf-1 in vivo. On the other hand, SMG-7, which recruits the SMG-5-SMG-7-Upf3aS complex to phosphorylated Upf-1, can interact with the N-terminal half of SMG-1 (7). As for Upf1 and Upf2, we have overexpressed them with SMG-1 in HEK293T cells and evaluated the interaction by immunoprecipitation. As shown in Supplementary Fig. 3*A*, *B*, and C, all the mutants showed the ability to interact with Upf1 and Upf2. These results and our previous study (7) suggested that both N-terminal and C-terminal regions of SMG-1 are involved in the interaction with Upf1 and C-terminal region except insertion region might be important for interaction with Upf2 *in vivo*.

We next examined the interaction between SMG-1 and SMG-7. As for SMG-7, we measured the interaction of overexpressed SMG-1 mutants and endogenous SMG-7 because we have an excellent antibody to SMG-7 (6). Interestingly, the interaction with SMG-7 was reduced in Δ1-152 and further reduction of the interaction was observed in Δ1-617; however, other mutants (Δ912-1014, Δ1675-1708, Δ2513-3580) did not change the affinity for SMG-7 (Fig.6). This result indicated that the N-terminal end of SMG-1 is required for the interaction with SMG-7.

Discussion

Analyses of kinase activity of SMG-1 mutants *in vitro* and *in vivo* revealed the specific structural features of SMG-1 and the common conserved features for PIKKs.

First, we found that a single point mutation, L3646A, in the FATC domain strongly affected SMG-1 kinase activity (Fig. 1*C* and *D*, and Fig 2), suggesting that the FATC domain probably locates close to the catalytic domain spatially. Furthermore, the fact that the large insertion region between the PIKK domain and the FATC domain was not critical for SMG-1 kinase activity *in vitro* (Fig. 3) may indicate that the large insertion region is not critical for the orientation between the PIKK and the FATC domains. This is consistent with the fact that lengths of insertion regions of SMG-1 are not conserved among species (Supplementary Fig. 1*A*) (1,3,20). The substrate-induced conformational change between the PIKK and FATC domains was also suggested for the case of DNA-PKcs, based on the electron microscopy (EM) observations (23,24). Other reports showed that the FATC domain was necessary for basal kinase activity in mTOR and DNA-PKcs, although not in ATM (15-17). A

drastic effect of the single amino acid mutation in the FATC domain of SMG-1 on an intrinsic kinase activity of SMG-1 suggests that role of the FATC domain for SMG-1 kinase activity is similar to those for mTOR and DNA-PKcs. Among the conserved amino acids in the FATC domains of PIKKs, L3646 was essential for SMG-1 kinase activity, but W3653 was not (Figs.1 and 2). The L3646A mutation of SMG-1 might disrupt the folding of the FATC domain itself since the corresponding leucine in yeast TOR1 (L2459) is required to make the correct folding of the FATC domain together with other hydrophobic and aromatic residues (25). The essential role of corresponding leucine in ATM (L3045) for its ionized radiation (IR) inducing activation (17) suggests that the possible contribution of L3646 resides for the regulation of SMG-1 kinase activity by extracellular stimulation, such as IR (8), besides basal kinase activity. Another conserved amino acid, W1962, in the TS domain, weakly affected SMG-1 kinase activity (Fig. 2), whereas the corresponding amino acid in mTOR, i.e., W2027, was critical for kinase activity (22). There is no evidence that SMG-1 interacts with FKBP12-rapamycin-binding complex that interacts with mTOR in the TS domain; however, the TS domain might regulate SMG-1 kinase activity similar to mTOR by binding to other unknown proteins or lipids.

Second, we showed that N-terminal sequences were required for kinase activity of SMG-1. The Δ1-617 mutant, which lacks most of OCR1, showed significantly reduced kinase activity compared to WT (Fig. 4*A*). Although the Δ1-617 mutant which contains all sequences of the isoform, as reported by Denning *et. al.* and Brumbaugh *et al.* (amino acids 626-3657; 340 kDa) (2,8), showed detectable kinase activity, its autophosphorylation activity was only 4.5% of WT, which was significantly higher than that of D2331A (1.5% of WT) ($p<0.005$, Student's *t* test; Fig. 4*A*, white bar). We could not detect any signals of endogenous SMG-1 except for 430-kDa (Fig. 5 *A* black arrowhead) and 400-kDa (Fig. 5 *A* white arrowhead) bands using our antibody against 4 independent regions of SMG-1 in any cell lines and tissues we tested (3), and the Δ1-617 band was significantly smaller than 400 kDa (Fig. 5*A*). Thus, we suggest that the isoform reported previously is not the 400-kDa isoform of SMG-1, and is a minor isoform that possesses little kinase activity, if existing. However, we did not exclude the possibility that the splice variant might be expressed in some specific cell phases and/or after stimulation.

Detailed truncation and internal deletion analyses of the N-terminal region of SMG-1 revealed that the whole N-terminal region was required for SMG-1 kinase activity. N-terminal halves of PIKKs are comprised of tandem helical repeats (Supplementary Fig. 1*B*) (18,19) and these helical repeats mediate interactions with distinct binding partners (26-28). However, there is little evidence whether this region is involved in PIKKs' intrinsic kinase activity. N-terminal truncation analyses of mTOR and ATM showed that deletion of more than 1000 amino acids far from the catalytic domain, containing tandem helical repeats, did not seriously affect kinase activity (22,29). However, in SMG-1, a small deletion in the N-terminal region strongly affected kinase activity *in vitro* and *in vivo* (Figure 4). Since immunopurified SMG-1 proteins used for the kinase assay showed no signs of contamination by other proteins (Fig. 1*B*), the cause of inactivation observed for many of the SMG-1 mutants didn't seem to involve failure in association with unknown accessory proteins. Therefore, we suggest that N-terminal region of SMG-1 is required for maintaining the proper conformation of the catalytic PIKK domain. Note that all the mutants tested, except for Δ1-152 and Δ1-617, could associate with known direct partner proteins, Upf1, Upf2, and SMG-7, supporting that they retains the ability to interact with these proteins.

Three-dimensional analyses of DNA-PKcs also revealed that the N-terminal regions of DNA-PKcs were located near the C-terminal region containing the PIKK domain and the FATC domain (23,24,30) and this conformation changed in response to binding to DNA and Ku subunit. In addition, X-ray crystal structure of the catalytic subunit of the class IB γ isoform of PI3K (PI3Kγ), whose catalytic domain is similar to PIKK domain, revealed that the helical domain akin to HEAT repeat provides a core scaffold, the surface of which interacts with other domains including catalytic domain (31,32). In this case, such folding manner makes an N-terminal Ras-binding domain, which is distantly positioned from the catalytic domain in the amino acid sequence position, a close contact to the catalytic domain, enabling a Ras-induced PI3K activation. A mutation of the helical domain of class Ia α

isoform of PI3K (PI3Kα), the structure of which is similar to PI3Kγ, affects the kinase activity of PI3Kα (33). Taken together with our biochemical study and the three-dimensional analyses of DNA-PKcs and PI3K, we suggest that the N-terminal region of SMG-1 would locate near the catalytic domain, and such specific conformation might be required for an intrinsic kinase activity of SMG-1. The catalytic domains of most protein kinases transit between an active open state and an inactive closed state, which are formed by N-terminal and C-terminal lobes of catalytic domain (34-36). We suggest that the N-terminal region of SMG-1 would modulate folding structure of the catalytic domain. Our findings that the N-terminal region is required for SMG-7 and presumably for Upf1 bindings support the notion that the large N-terminal domain forms a large scaffold for other accessory proteins and regulates the catalytic activity of SMG-1. SMG-7 is required for recruiting the SMG-5-SMG-7-PP2A complex to the phosphorylated Upf1 (6). Our recent study has also revealed that SMG-7 can interact with SMG-1-Upf1 complex in the absence of the phosphorylation of Upf1 (7). Thus, it is possible that SMG-7 can have dual activities, recruitment of PP2A phosphatase complex to phosphorylated Upf1 and suppression of SMG-1 catalytic activity through the interaction with the N-terminal domain.

A recent study reported that the N-terminal region of mTOR was required for multimerization in response to nutrients (37). We also observed multimerization of SMG-1 via both the N-terminal and C-terminal regions (Supplementary Fig. 4*A* and *C*). However, this multimerization became undetectable in the presence of 1% Triton X-100 (Supplementary Fig. 4*B*), a similar condition for our immunopurification and kinase assays. Thus, multimerization may not be related to SMG-1 kinase activity as far as our assay conditions were concerned, although we could not exclude the possibility that multimerization might regulate SMG-1 kinase activity or affinity to other proteins *in vivo*. Although autophosphorylations of ATM and DNA-PKcs have been reported to be involved in regulation of their activities (9,38,39), a role of autophosphorylation of SMG-1 observed in this study still remains unclear because accelerated autophosporylation of Δ2513-3490 did not significantly affect the kinase activity.

We found that all mutants tested for Upf1 phosphorylation *in vivo* showed kinase activity *in vitro* (Fig.5). We could not compare the kinase activity between i*n vivo* and *in vitro* accurately because the amount of SMG-1 mutants in Fig.5 could not be measured accurately due to the difference of molecular weight, although purified SMG-1 proteins could be measured in *in vitro* kinase assay (Fig. 1-4). However, the mutants, which possessed kinase activity *in vitro,* retained the ability to phosphorylate Upf1 more than kinase inactive mutant did. We failed to obtain SMG-1 mutants with intrinsic kinase activity, but without interaction with other essential components. For example, Upf2 binds to the C-terminal region of SMG-1 (7), and our previous study established that *in vivo* phosphorylation of Upf1 required interaction between SMG-1 and Upf2 (7). However, all mutants retained interaction with Upf2 (Supplementary Fig. 3*C*).

In conclusion, our extensive mutational analysis of SMG-1 revealed a novel aspect into nature of the basal kinase activity of SMG-1, the newest member of the PIKK family. Characterization of SMG-1 kinase activity will provide important clues for understanding the regulation of SMG-1 kinase activity and mRNA surveillance.

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Footnotes

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The abbreviations are: SMG, suppressor of morphogenetic effect on genitalia; Upf, up frameshift; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; PP2A, protein phosphatase 2A, PIKK, phosphatidylinositol 3-kinase-related protein kinase; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3- related; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; TRRAP, transformation/transcription domain-associated protein; FATC, FRAP ATM TRRAP C-terminal; FRB, FKBP12-rapamycin binding; FRBH, FRB homology; OCR, one-specific conserved region; TPR, tetraticopeptide repeat; ARM, armadillo repeat motif; PCR, polymerase chain reaction; WT, wild type; GST, glutathione *S*-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium.

Figure legends

Fig. 1. Dose-dependent SMG-1 kinase activity
(A) Schematic representation of domains in human SMG-1. The TS domain, PIKK domain, and FATC domain are indicated. In addition, the 3 homologous regions between human SMG-1 and *C.elegans* SMG-1 (OCR1, OCR2, and OCR3) are indicated. Numbers represent amino acid locations from the N-terminus. (B) To measure amount of FLAG-immunopurified FLAG-SMG-1 proteins, immunopurified proteins were separated by 7% SDS-PAGE with BSA (lanes 1-5) as standard. Following silver staining, the amount of FLAG-SMG-1 WT (lanes 7 and 8) and FLAG-SMG-1 D2331A (lanes 9 and 10) were calculated using the BSA calibration curve. Asterisks indicate nonspecific bands that may reflect BSA monomers and dimers that are used in the blocking FLAG-affinity resin. (C) 20ng to 300 ng immunopurified FLAG-SMG-1 proteins were subjected to *in vitro* kinase assays with GST- $Upf1^{1072\text{-}1085}$ as a substrate in the presence of [γ-^{32}P] ATP. Asterisks indicate autophosphorylation of FLAG-SMG-1 proteins, and arrowheads indicate phosphorylated substrates. (D) Results in (C) were quantified by densitometric analysis with the Image Gauge software, and were plotted accordingly. Values represent means ± SD from 3 independent trials.

Fig. 2. Important amino acids for SMG-1 kinase activity in the TS and FATC domains. (A)

Kinase activity of FLAG-SMG-1 point mutants. HEK293T cells were transfected with either an empty vector, FLAG-SMG-1 WT, FLAG-SMG-1 D2331A, FLAG-SMG-1 W1962F, FLAG-SMG-1 L3646A or FLAG-SMG-1 W3653F expression vector. Anti-FLAG immunoprecipitates were subjected to an *in vitro* kinase assay with GST- Upf1$^{1072\text{-}1085}$ as substrate, in the presence of [γ-^{32}P] ATP. Recombinant SMG-1 levels were determined by immunoblotting with anti-FLAG antibody, and substrates were detected by autoradiography, followed by CBB staining. (B) Results in (A) were quantified by densitometric analysis with the Image Gauge software, and were normalized against data for the FLAG-SMG-1 WT-transfected sample. Black bar indicates the kinase activity of SMG-1 proteins for GST- Upf1$^{1072\text{-}1085}$ and white bar indicates autophosphorylation levels of SMG-1 proteins. Values represent mean ± SD from 4 independent trials.

Fig. 3. The insertion region between the PIKK and FATC domains of SMG-1 is not critical for kinase activity. Kinase activities of FLAG-SMG-1 insertion region deleted mutants. The left panel is a schematic structure of deletion mutants. The right panel represents kinase activity levels of deletion mutants from an *in vitro* kinase assay, as described in Fig. 2. Recombinant SMG-1 levels were determined by silver staining and substrates were detected by autoradiography, followed by CBB staining. Black bar indicates the kinase activity of SMG-1 proteins for GST- Upf1$^{1072\text{-}1085}$ and white bar indicates autophosphorylation levels of SMG-1 proteins. Values represent mean ± SD from 3 independent trials.

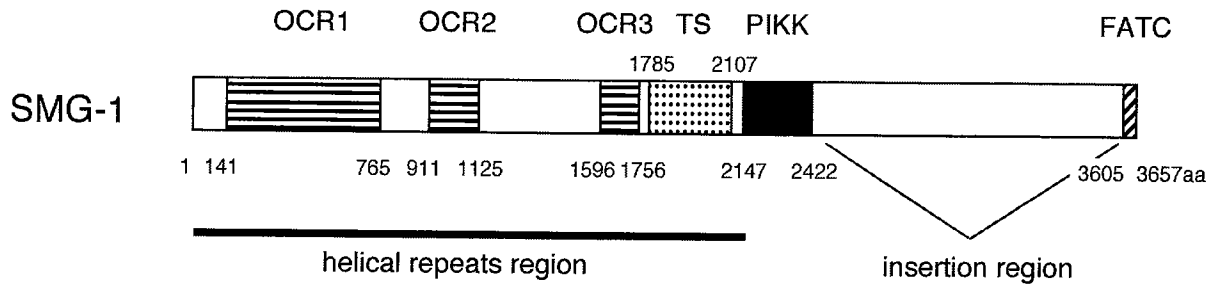
Fig. 4. The N-terminal region of SMG-1 is essential for kinase activity. (A) Kinase activities of FLAG-SMG-1 sequential N-terminal deletion mutants. The left panel is a schematic structure of deletion mutants. The right panel represents kinase activity levels of deletion mutants from an *in vitro* kinase assay, as described in Fig. 2. Recombinant SMG-1 levels were determined by silver staining and substrates were detected by autoradiography, followed by CBB staining. Values represent mean ± SD from 3 independent trials. (B) Kinase activities of FLAG-SMG-1 mutants that lack short amino acid sequences in the N-terminal region. The left panel is a schematic structure of deletion mutants, and the right panel represents kinase activity levels of deletion mutants from an *in vitro* kinase assay, as described above. Black bar indicates the kinase activity of SMG-1 proteins for GST- Upf1$^{1072\text{-}1085}$ and white bar indicates autophosphorylation levels of SMG-1 proteins. Values represent mean ± SD from 3 independent trials.

Fig. 5. *In vivo* Upf1 phosphorylation activity of SMG-1 mutants. (A) HeLa TetOff cells transfected with siRNA against SMG-1 3'UTR together with the indicated FLAG-SMG-1 mutants were separated by 6 % SDS-PAGE; and FLAG-SMG-1 mutants, endogenous Upf1 and phosphorylated Upf1 were detected by Western blotting. Black and white arrowheads indicate endogenous SMG-1 isoforms of 430 kDa and 400 kDa, respectively. Asterisks indicate exogenous FLAG-SMG-1 proteins. (B) Amount of phosphorylated Upf1 was normalized against total Upf1 and graphed. Values represent mean ± SD from 3 independent trials.

Fig. 6. Interaction of FLAG-SMG-1 mutants with SMG-7.

HEK293T cells expressing the indicated FLAG-SMG-1 mutants and were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.4% Nonidet-P40, 200 μg/ml RNase A, 100 nM okadaic acid, and protease inhibitors; and were immunoprecipitated with anti-HA antibody. After immunoprecipitates were separated by 7% SDS-PAGE, FLAG-SMG-1 mutants and endogenous SMG-7 (short isoform and long isoform) were detected by Western blotting using anti-FLAG (Sigma) and anti-SMG-7 (6) antibodies.

A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OCR2
OCR3
TS
PIKK
FATC
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SMG-1
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765 911 1125
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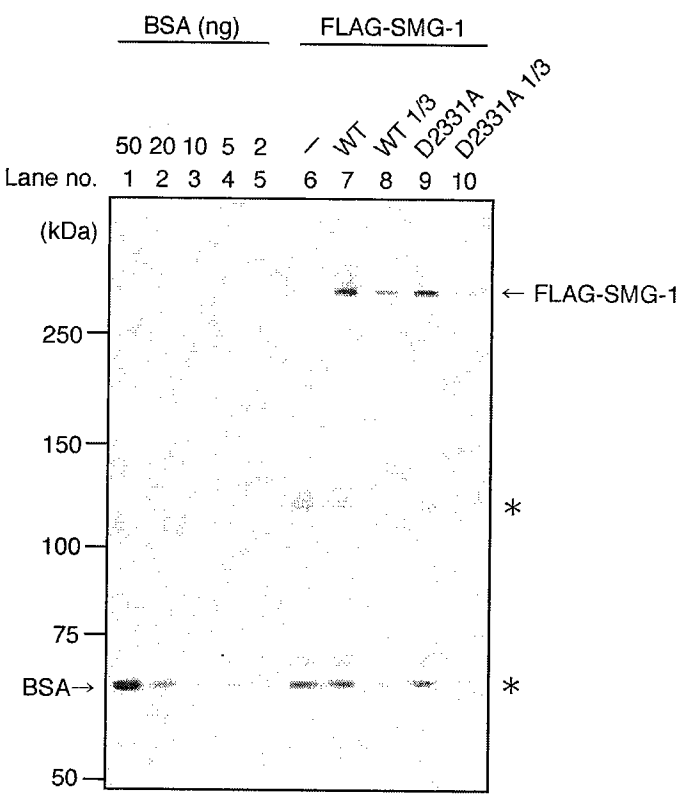
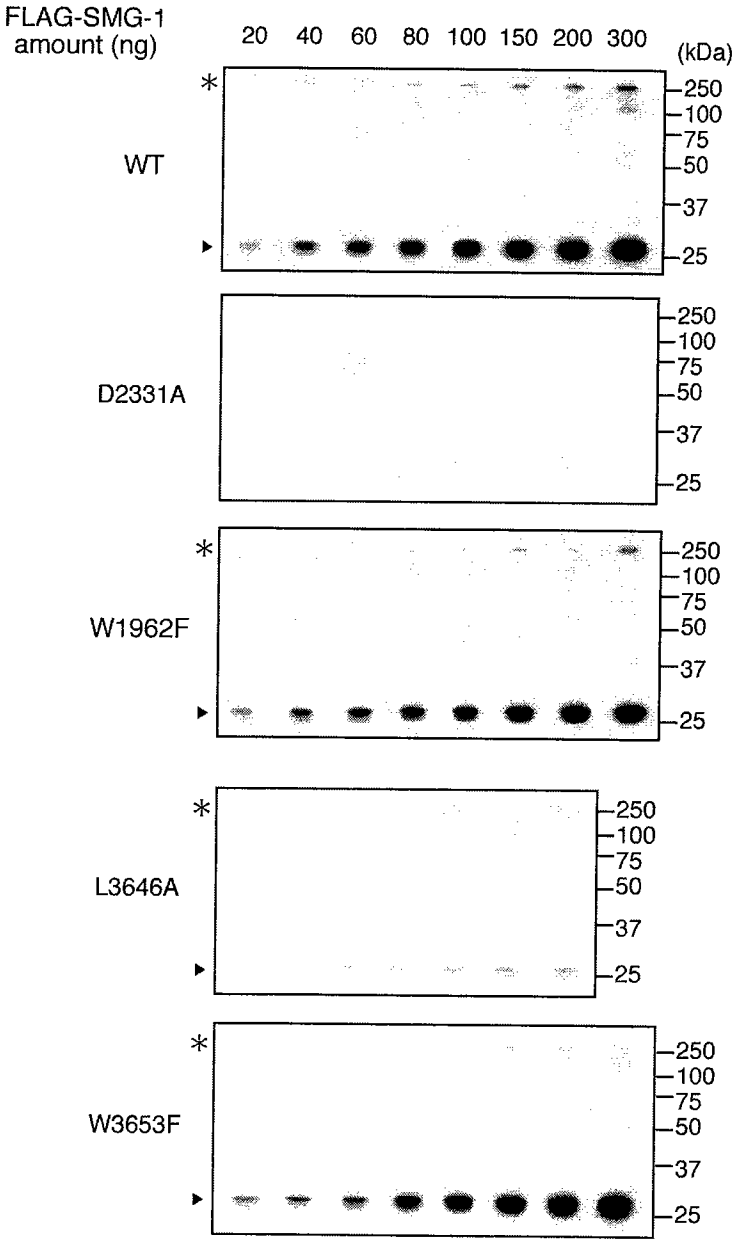
2147 2422
3605 3657aa
helical repeats region
insertion region

B


BSA (ng)
FLAG-SMG-1
50 20 10 5 2
/
WT
WT 1/3
D2331A
D2331A 1/3
Lane no. 1 2 3 4 5 6 7 8 9 10
(kDa)
250
150
100
75
BSA→
50
← FLAG-SMG-1
*
*

C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(kDa)
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W1962F
L3646A
W3653F
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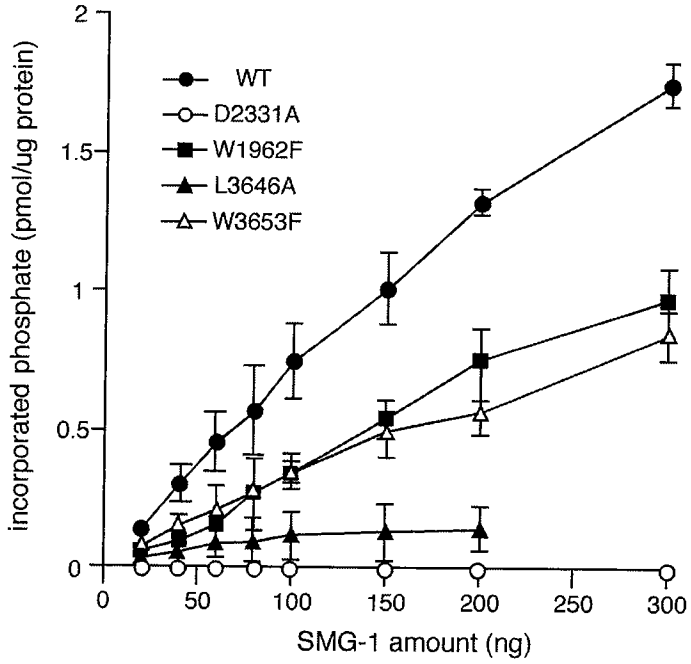
100
75
50
37
25

Figure 1

D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SMG-1 amount (ng)
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D2331A
W1962F
L3646A
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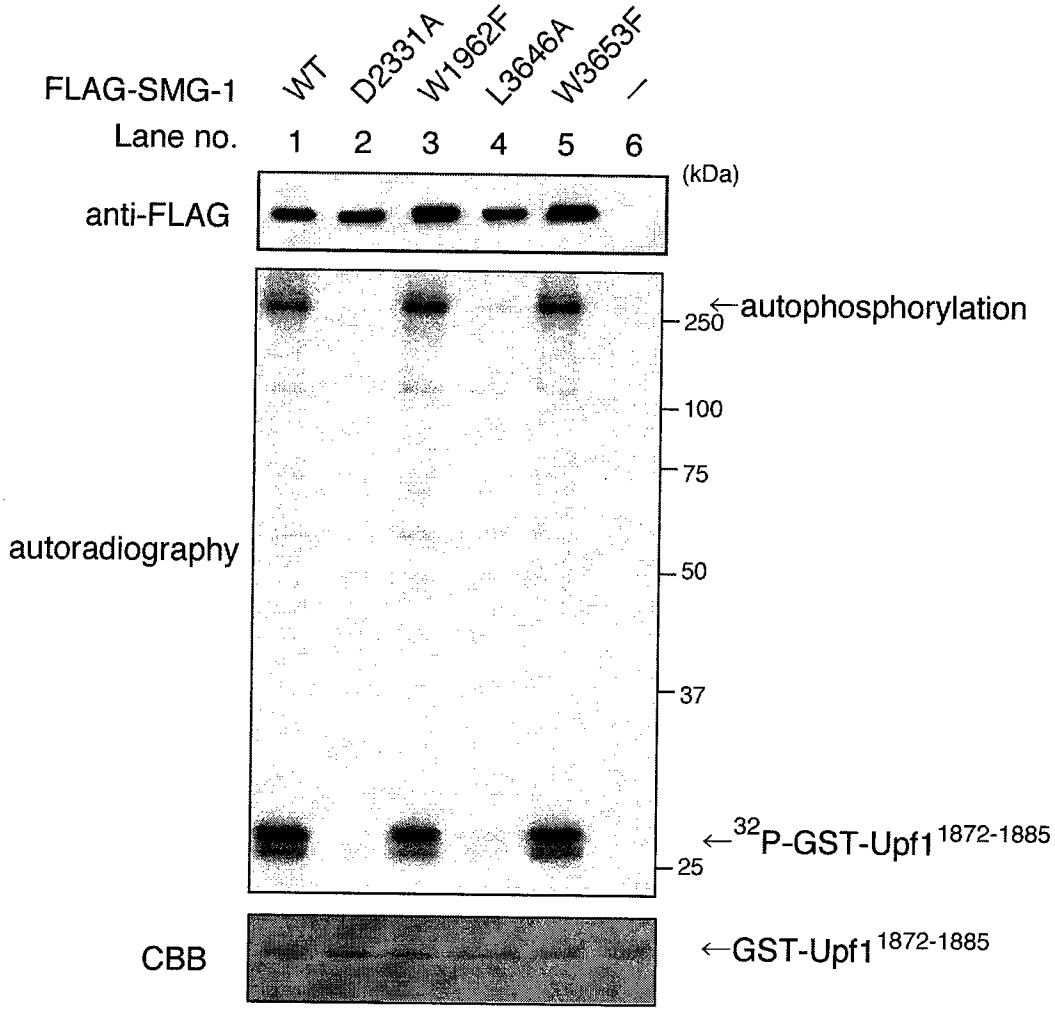
0.5
1
1.5
2
0
50
100
150
200
250
300

Figure 2

A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D2331A
W1962F
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W3653F
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Lane no.
1 2 3 4 5 6
(kDa)
anti-FLAG
autoradiography
250
100
75
50
37
25
←autophosphorylation
←32P-GST-Upf1 1872-1885
CBB
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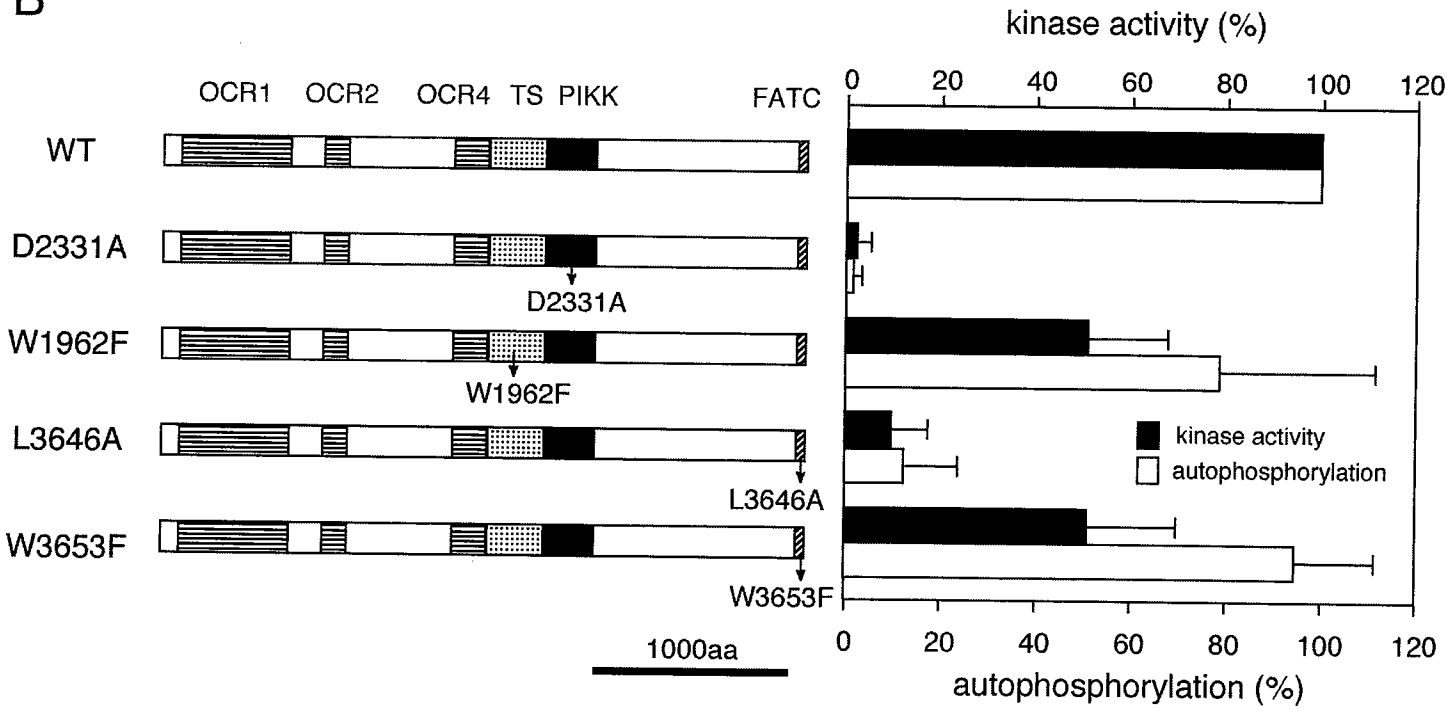
B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PIKK
FATC
WT
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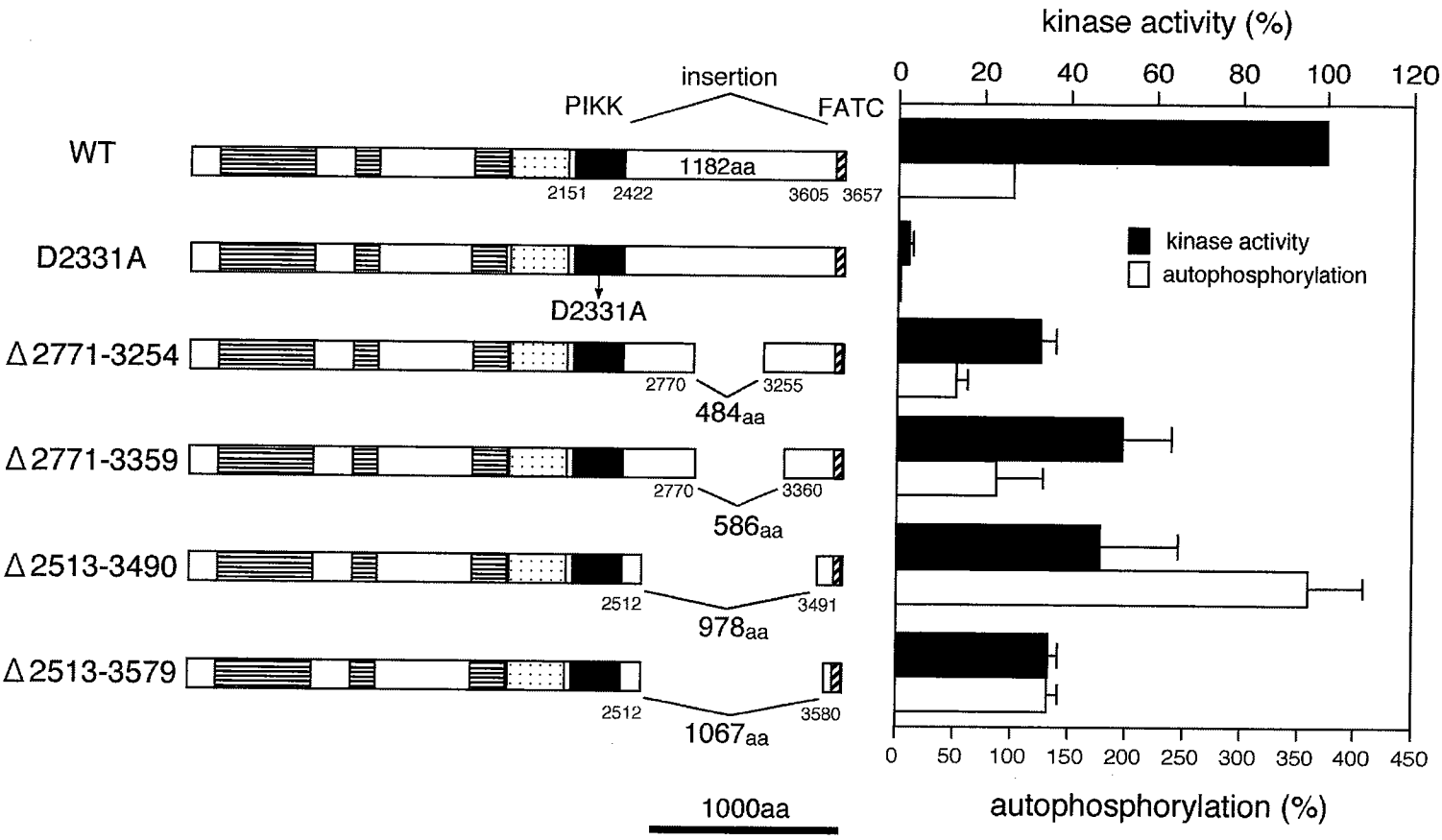
kinase activity (%)
0 20 40 60 80 100 120
kinase activity
autophosphorylation
autophosphorylation (%)

Figure 3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D2331A
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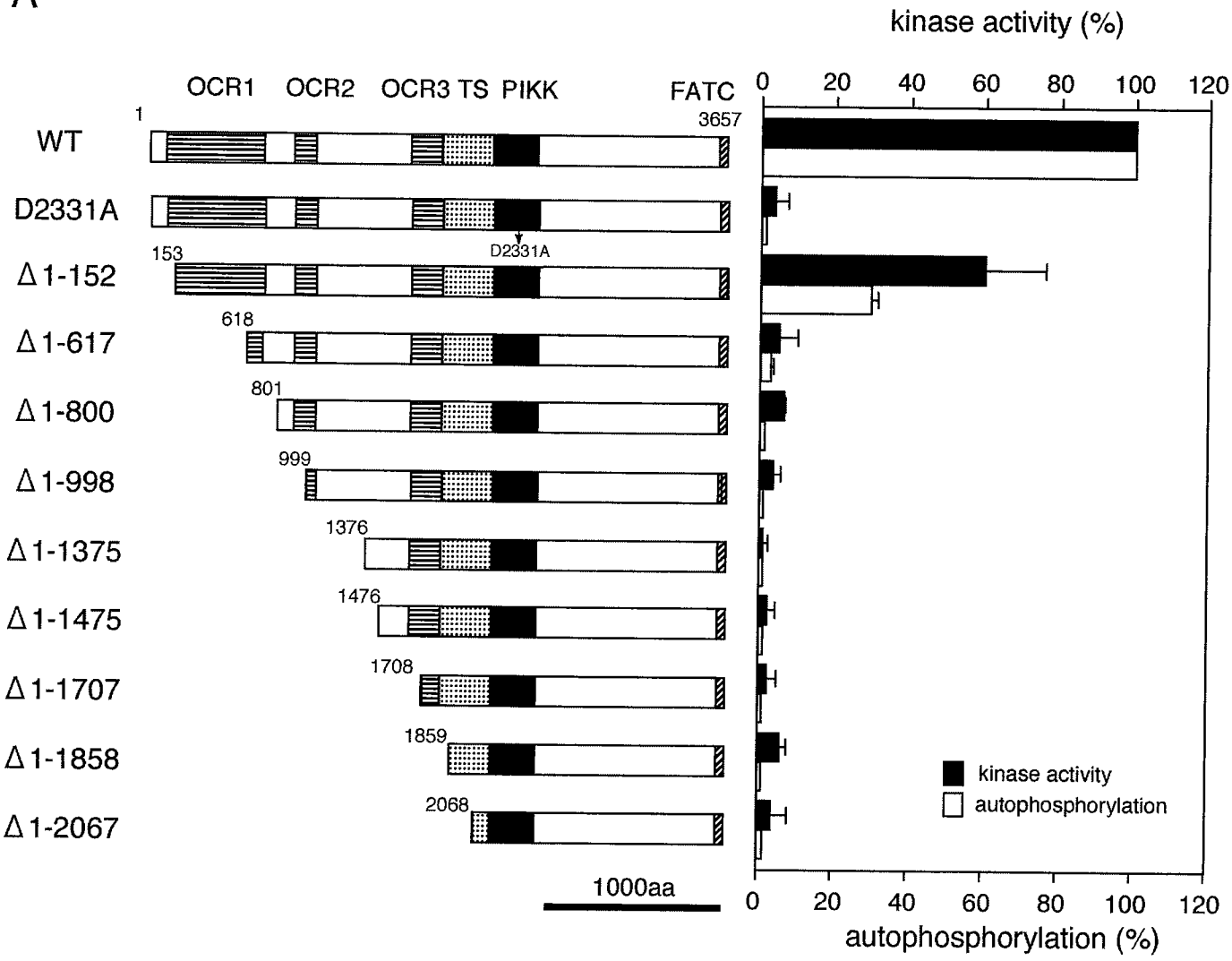
3255
484aa
Δ2771-3359
2770
3360
586aa
Δ2513-3490
2512
3491
978aa
Δ2513-3579
2512
3580
1067aa
1000aa
kinase activity (%)
0
20
40
60
80
100
120
kinase activity
autophosphorylation
0
50
100
150
200
250
300
350
400
450
autophosphorylation (%)

Figure 4

A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WT
D2331A
153
D2331A
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618
Δ1-617
801
Δ1-800
999
Δ1-998
1376
Δ1-1375
1476
Δ1-1475
1708
Δ1-1707
1859
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2068
Δ1-2067
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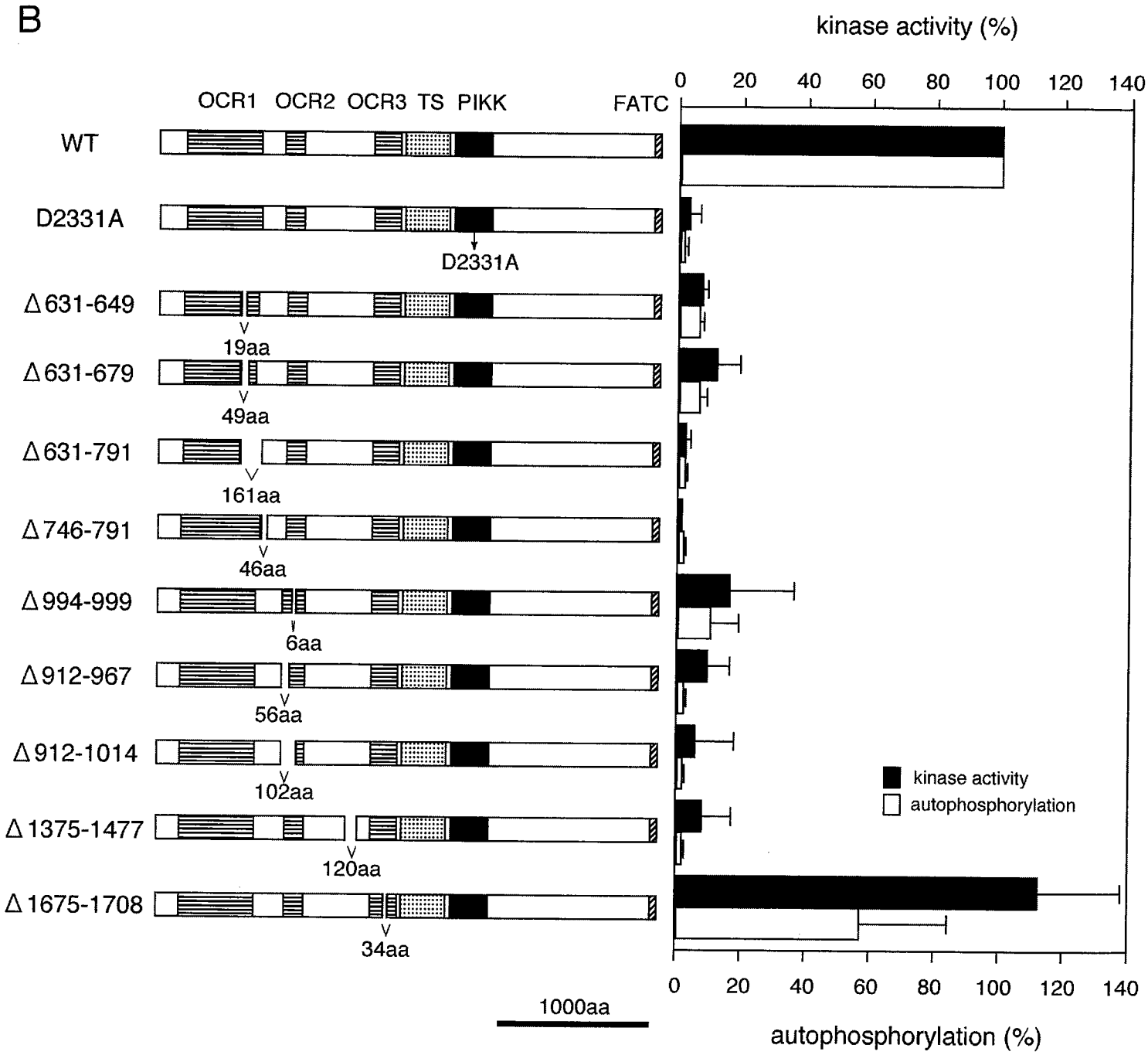
kinase activity
autophosphorylation
0 20 40 60 80 100 120
autophosphorylation (%)

Figure 4


B
kinase activity (%)
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D2331A
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49aa
Δ631-791
161aa
Δ746-791
46aa
Δ994-999
6aa
Δ912-967
56aa
Δ912-1014
102aa
Δ1375-1477
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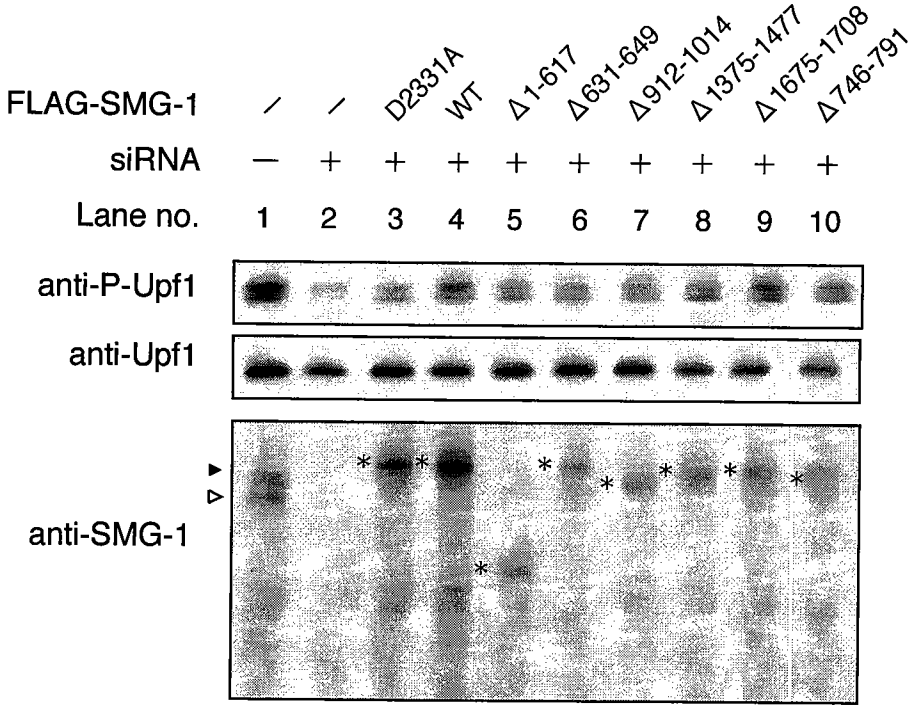
34aa
1000aa
kinase activity
autophosphorylation
0 20 40 60 80 100 120 140
autophosphorylation (%)

Figure 3

A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Lane no.
anti-P-Upf1
anti-Upf1
anti-SMG-1
/ / D2331A WT Δ1-617 Δ631-649 Δ912-1014 Δ1375-1477 Δ1675-1708 Δ746-791
— + + + + + + + + +
1 2 3 4 5 6 7 8 9 10

B

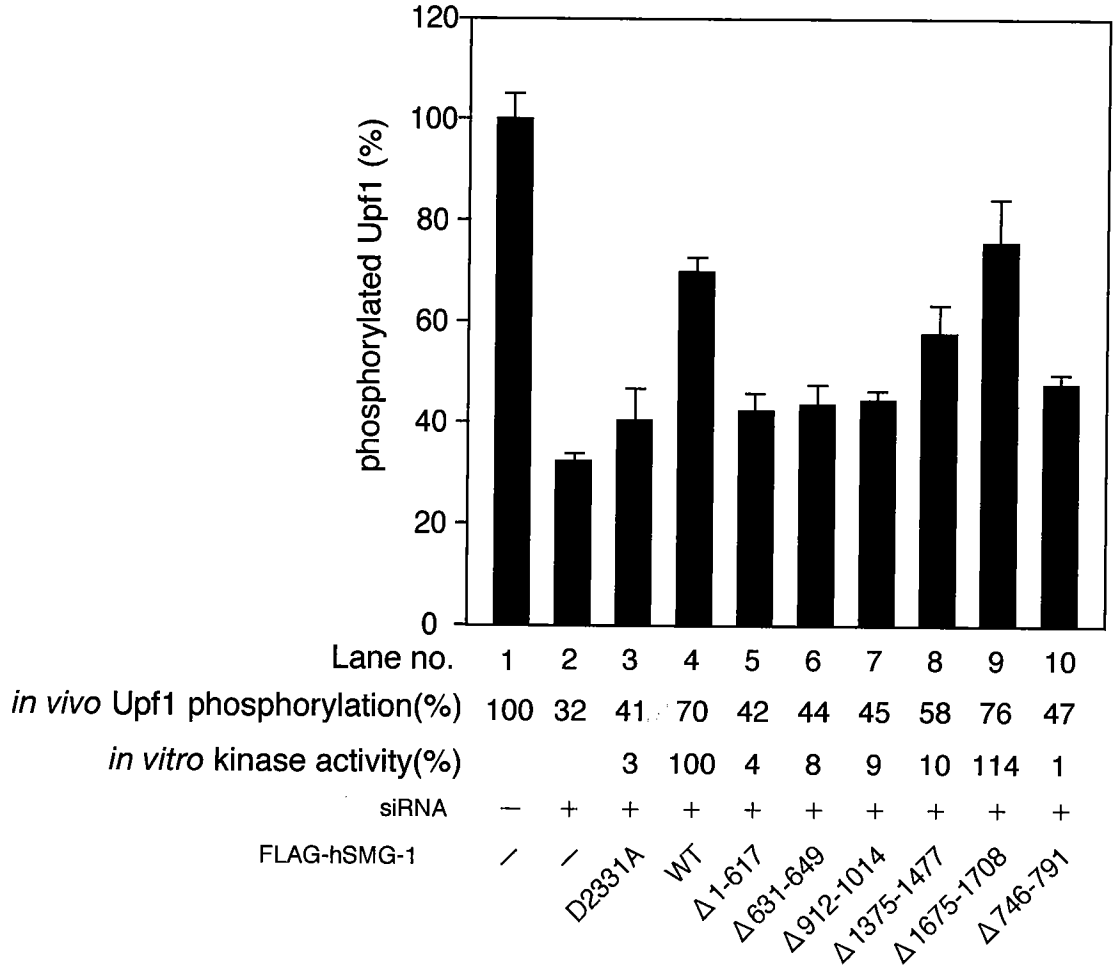
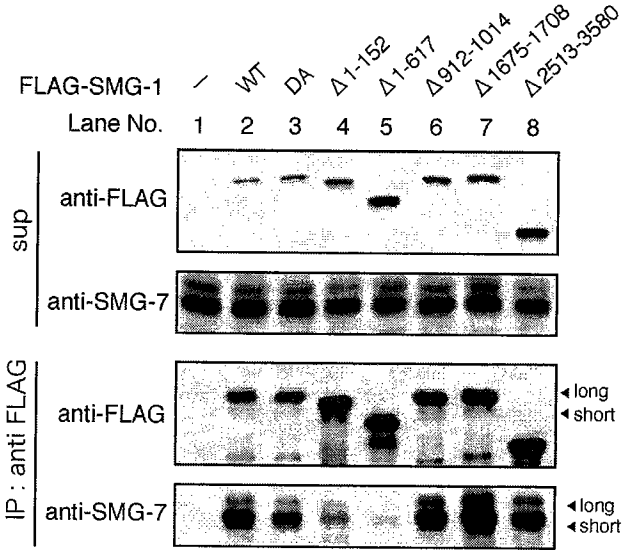

phosphorylated Upf1 (%)
120
100
80
60
40
20
0
Lane no. 1 2 3 4 5 6 7 8 9 10
in vivo Upf1 phosphorylation(%) 100 32 41 70 42 44 45 58 76 47
in vitro kinase activity(%) 3 100 4 8 9 10 114 1
siRNA — + + + + + + + + +
FLAG-hSMG-1 / / D2331A WT Δ1-617 Δ631-649 Δ912-1014 Δ1375-1477 Δ1675-1708 Δ746-791

Figure 6


FLAG-SMG-1
–
WT
DA
Δ1-152
Δ1-617
Δ912-1014
Δ1675-1708
Δ2513-3580
Lane No.
1
2
3
4
5
6
7
8
sup
anti-FLAG
anti-SMG-7
IP : anti FLAG
anti-FLAG
anti-SMG-7
long
short
long
short